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14. ABSTRACT

In the past year significant progress has been made towards completing tasks both outlined in and extending beyond the original Statement of Work. The physical association between XIAP and AIF was determined to be highly dependant upon the ubiquitination status of AIF, and XIAP-mediated ubiquitination of AIF was shown not to result in AIF degradation. It was determined that the XIAP antagonist Smac/DIABLO completely disrupted the association between XIAP and AIF, in a manner that correlated with an increase in AIF ubiquitination. AIF was determined to be a substrate for cleavage by the serine protease and XIAP antagonist Omi/HtrA2, yet the absence of Omi/HtrA2 did not affect the cytoplasmic release of AIF following apoptosis induction. Interestingly, the release of cytochrome c following cell death induction was shown to be delayed in cells deficient in Omi/HtrA2. Finally, AIF was found to associate with the XIAP homologues cIAP-1 and cIAP-2, suggesting that AIF may be a general purpose IAP binding protein. These findings represent significant progress in further characterizing the interaction between AIF and the inhibitor of apoptosis (IAP) family, and continue to form the basis for understanding how AIF and the IAP family contribute to the pathogenesis of prostate cancer.

15. SUBJECT TERMS
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Introduction

Prostate cancer is the second most common form of cancer diagnosed among men in the western world, representing a significant healthcare threat. Difficulties in prevention and treatment of prostate cancer are due in part to the lack of reliable biomarkers to predict disease stage and behavior, as well as a lack of understanding at the molecular level as to the events giving rise to prostate tumorigenesis and metastasis. My original research proposal outlined studies aimed at understanding how two well known regulators of programmed cell death, X-linked inhibitor of apoptosis (XIAP) and apoptosis inducing factor (AIF), contribute to the overall pathogenesis of prostate cancer. This current report summarizes progress made in the second year towards completing the research proposal goals.

Body

In year one, significant progress was made toward completing the tasks outlined in my original Statement of Work, such that several aims were taken to completion. In year two, additional significant progress has been made, as summarized below:

AIM 1. To determine the effects of AIF on XIAP function.

a) **Characterization of the XIAP/AIF interaction under normal and apoptotic conditions.** We have found that the interaction between XIAP and the apoptotic form of AIF (Δ102) is highly dependant on the E3 ubiquitin ligase activity of XIAP. I have determined that whereas wildtype XIAP has only a weak affinity for Δ102 AIF, the XIAP variant H467A, which lacks the ability to ubiquitinate target molecules, is significantly enhanced in its ability to bind AIF (Figure 1). These data suggest that there is a transient, ubiquitin dependant association between XIAP and AIF, and that XIAP-mediated AIF ubiquitination may function to regulate the signaling properties of the AIF molecule. Further, I have determined that proteasomal inhibition does not result in an increase in endogenous AIF protein levels, and that XIAP-mediated ubiquitination of AIF does not result in AIF degradation (Figure 2). Taken together, these data suggest that the polyubiquitin chain formation that occurs on the AIF protein is mediated through lysine residues of ubiquitin other that K48, the predominant residue for targeting to the proteasome. In a separate line of investigation, I have examined the ability of the XIAP antagonist Smac/DIABLO to displace AIF from interaction with XIAP, and have determined that the overexpression of Smac/DIABLO prevents AIF from associating with XIAP (Figure 3). Examination of cellular lysates indicates that Smac/DIABLO overexpression results in an increase in the presence of high molecular weight AIF species, consistent with an increase in the amount of ubiquitinated AIF material. These data further support the hypothesis that the interaction between XIAP and AIF is highly dependant upon AIF ubiquitination status.
b) **Assessment of the effects of AIF on the caspase inhibitory properties of XIAP.** This sub-aim was completed in year one.
c) **Assessment of the effects of AIF on the ability of XIAP to augment TGF-β, JNK, or NF-κB signaling.** Experiments are in progress.
d) **Evaluation of the effects of AIF on XIAP function in prostate tumor-derived cell lines.** Experiments are in progress.
e) **Characterization of the effects of AIF on the copper metabolism regulatory properties of XIAP.** We have found that the binding of copper by XIAP, which results in a profound conformational change within the XIAP protein (Mufti et al, 2006), does not affect the ability of XIAP to bind AIF (Figure 4). Further experiments are in progress.

AIM 2. To determine the effects of XIAP on AIF function.

a) **Assessment of the effects of XIAP on the death promoting properties of AIF.** This sub-aim was completed in year one.
b) **Determination of the caspase-dependence of the interaction between XIAP and AIF.** This sub-aim was completed in year one.
c) **Assessment of the effects of XIAP on the NADH-oxidase activity of AIF (Months 14-18).** Experiments are in progress.
d) **Characterization of the ability of XIAP to ubiquitinate AIF.** This sub-aim was completed in year one. Further observations are reported above for Aim1a.

In addition to the progress highlighted above, I have made further discoveries that relate to the biological functions of XIAP and AIF, which are supplemental to the tasks outlined in my proposal. It has recently been reported that the release of AIF from the mitochondria during apoptosis requires two distinct events (Otera et al, 2005). The first is mitochondrial outer membrane permeablization (MOMP), a process that is regulated predominantly by members of the Bcl-2 family of proteins. The second is proteolytic cleavage by an as yet undefined protease, most likely of the serine protease family. I have determined that the XIAP antagonist Omi/HtrA2, which possesses serine protease activity, is capable of cleaving the AIF protein in vitro (Figure 5). Given the ability of XIAP to interact with both Omi/HtrA2 and AIF, this raised the possibility that XIAP regulates the second step of AIF mitochondrial release, potentially through regulating the activity of Omi/HtrA2. To test this hypothesis, we investigated the release of AIF from the mitochondrial of mouse embryonic fibroblasts (MEFs) derived from mice deficient in Omi/HtrA2. When compared to control wildtype MEFs, no obvious differences were observed in the release of AIF from Omi/HtrA2 deficient cells (Figure 6). Interestingly, there was a significant reduction in the release cytochrome *c*.

Given the high degree of similarity between members of the inhibitor of apoptosis (IAP) family, of which XIAP is the best described member, the data presented characterizing the significant interaction between XIAP and AIF raised the possibility that AIF may bind other IAP family members. I have tested this possibility by examining the ability of AIF to bind the XIAP homologues cIAP-1 and cIAP-2, and have determined that both IAP proteins interact strongly with AIF (Figure 7), suggesting that AIF may be a general purpose IAP binding protein.

Key Research Accomplishments

- The generally weak interaction between XIAP and the apoptotic (Δ102) form of AIF was found to be stabilized when the E3 ubiquitin ligase activity of XIAP was disrupted
- Steady state AIF protein levels were shown to be unaffected by proteasomal inhibition, and XIAP-mediated ubiquitination of AIF was shown not to result in AIF degradation
- The AIF/XIAP interaction was shown to be disrupted by the overexpression of Smac/DIABLO, likely due to increased AIF ubiquitination
- Copper bound XIAP was determined to retain the ability to bind AIF
- AIF was shown to be a substrate for cleavage by the drosophila homologue of the IAP antagonist Omi/HtrA2, but no effects on the release of AIF from mitochondria were observed in Omi/HtrA2 deficient cells
- Delayed cytochrome C release was observed in cells deficient in Omi/HtrA2
- AIF was shown to robustly associate with the XIAP homologues cIAP-1 and cIAP-2

Reportable Outcomes

The following abstract was presented at the 2006 Gordon Conference on Cell Death in the last year:

- **Wilkinson, J.C.**, Wilkinson, A.S., and Duckett, C.S. AIF is an XIAP interacting protein and target for XIAP-mediated ubiquitination

Conclusions

Based on the progress in year two, current conclusions include: 1) the interaction between XIAP and AIF is dependant, in part, upon the ubiquitination status of AIF, 2) XIAP-mediated ubiquitination of AIF does not result in AIF degradation by the proteasome, 3) the XIAP antagonist Smac/DIABLO interferes with the ability of AIF to bind XIAP possibly through increasing AIF ubiquitination, 4) the copper bound form of XIAP retains the ability to bind AIF, 5) AIF is a substrate for cleavage by the serine protease/XIAP antagonist Omi/HtrA2, but mitochondrial release of AIF does not appear to depend on Omi/HtrA2 activity, 6) cytochrome *c* release is delayed in Omi/HtrA2 deficient cells, 7) AIF associates robustly with the XIAP homologues cIAP-1 and cIAP-2.

References

Mufti AR, Burstein E, Csomos RA, Graf PC, Wilkinson JC, Dick RD, Challa M, Son JK, Bratton SB, Su GL, Brewer GJ, Jakob U, & Duckett CS. XIAP Is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. *Molecular Cell* **21(6)**, 775-785 (2006).

Otera H, Ohsakaya S, Nagaura Z, Ishihara N, & Mihara K. Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. *EMBO J* **24(7)**, 1375-1386 (2005).

Appendix

Abstract presented at the 2006 Gordon Conference on Cell Death:

AIF IS AN XIAP INTERACTING PROTEIN AND TARGET FOR XIAP-MEDIATED UBIQUITINATION

John C. Wilkinson[1], Amanda S. Wilkinson[1], and Colin S. Duckett[1,2]. Departments of Pathology[1] and Internal Medicine[2], University of Michigan, Ann Arbor, MI, 48109

X-linked inhibitor of apoptosis (XIAP) is a well characterized inhibitor of cell death that protects cells by both caspase-dependent and independent mechanisms. In a biochemical screen for molecules that participate with XIAP in regulating cellular processes, we identified apoptosis inducing factor (AIF) as an XIAP binding protein. Domain analysis revealed that BIR2 of XIAP is both necessary and sufficient for interaction with AIF, and an XIAP variant incapable of binding caspases retained the ability to interact with AIF, suggesting that AIF employs a fundamentally different binding mechanism than other XIAP-associated factors. Human AIF localized to mitochondria, and the form present in healthy cells was determined to lack first 54 amino acids, differing significantly from the apoptotic form previously reported that lacks the first 102 amino-terminal residues. Fluorescence complementation and immunoprecipitation experiments revealed that XIAP is capable of interacting with both the healthy (delta 54) and apoptotic (delta 102) forms of AIF. Unlike other mitochondrial IAP antagonists, AIF failed to abrogate XIAP-mediated inhibition of caspase activity or prevention of apoptosis. Interestingly, AIF was found to be a target of XIAP-mediated ubiquitination, and an XIAP variant lacking E3 ubiquitin ligase activity displayed a more robust interaction with AIF. Expression of either XIAP or AIF reduced cellular ROS levels, and when expressed in combination, a synergistic decrease in both basal and peroxide-stimulated ROS was observed. Overall, these results identify AIF as a new XIAP binding partner, and suggest that in addition to its role in regulating caspase activation, XIAP regulates cellular ROS levels through interaction with AIF.

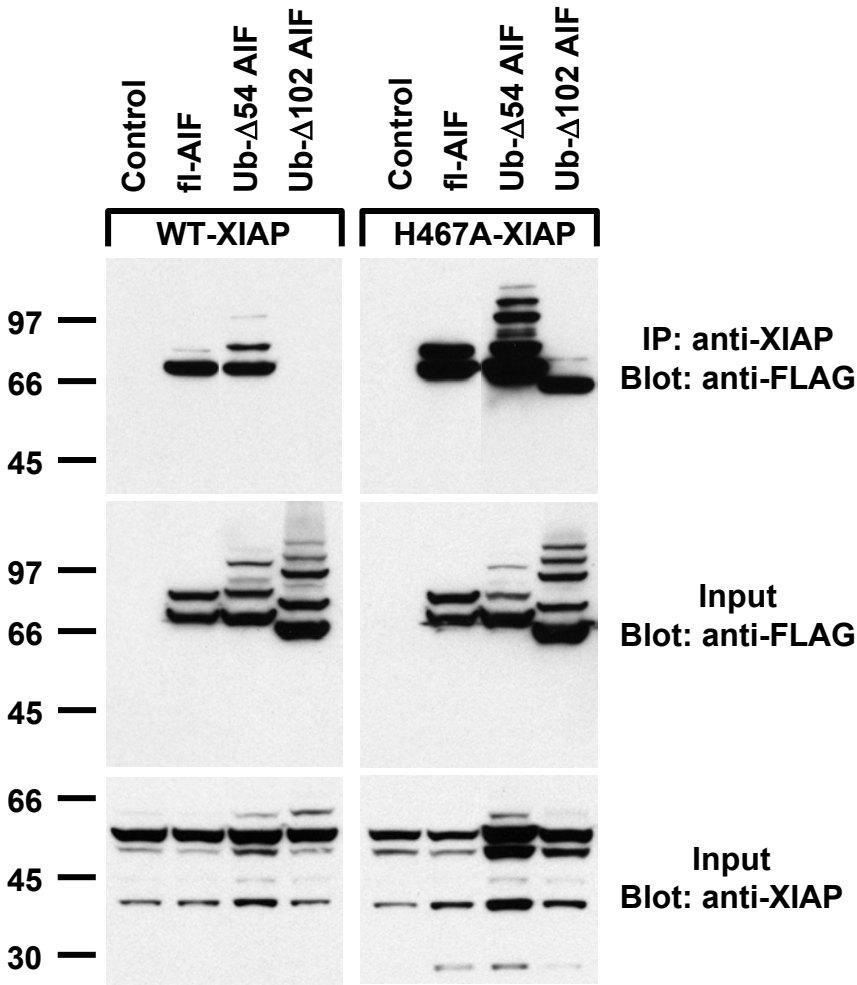

Figure 1: AIF association with wildtype, H467A XIAP. HEK 293 cells were transiently transfected with plasmids encoding the indicated proteins. Cellular lysates were prepared and XIAP was precipitated with an XIAP-specific antibody. The presence of AIF in precipitated complexes was determined by immunoblotting for the FLAG tag present at the carboxy terminus of each AIF protein. Note that both wildtype and the E3 ubiquitin ligase deficient (H467A) forms of XIAP robustly associate with full-length (fl) and Δ54 AIF, but only H467A-XIAP binds strongly to Δ102-AIF.

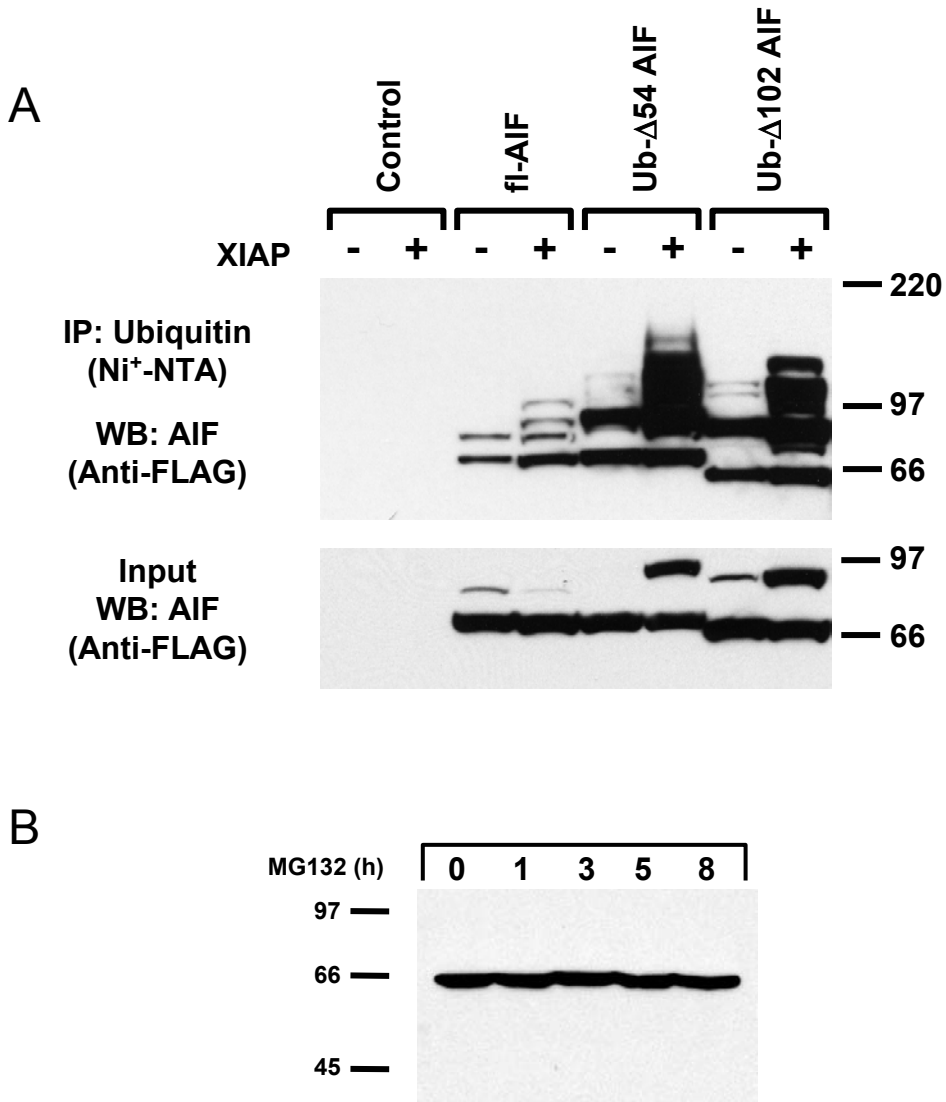

Figure 2: Ubiquitination of AIF does not result in degradation. Panel A: HEK 293 cells were transiently transfected with His-tagged ubiquitin and the indicated plasmids. Ubiquitinated material was then precipitated using Ni^{2+}-NTA beads, and the presence of FLAG-tagged proteins (AIF) in precipitated complexes (IP) was detected by immunoblot analysis (WB). Note that XIAP-mediated ubiquitination of AIF does not result in a decrease in recovered AIF protein (lower panel). Further note, this data was included in year one annual summary report, but is shown again here to highlight the lack of AIF degradation. Panel B: untransfected HEK 293 cells were treated with the proteasomal inhibitor MG132 for the indicated amounts of time. Cellular lysates were prepared and immunoblotted for the presence of AIF protein. Note the lack of AIF accumulation following MG132 treatment.

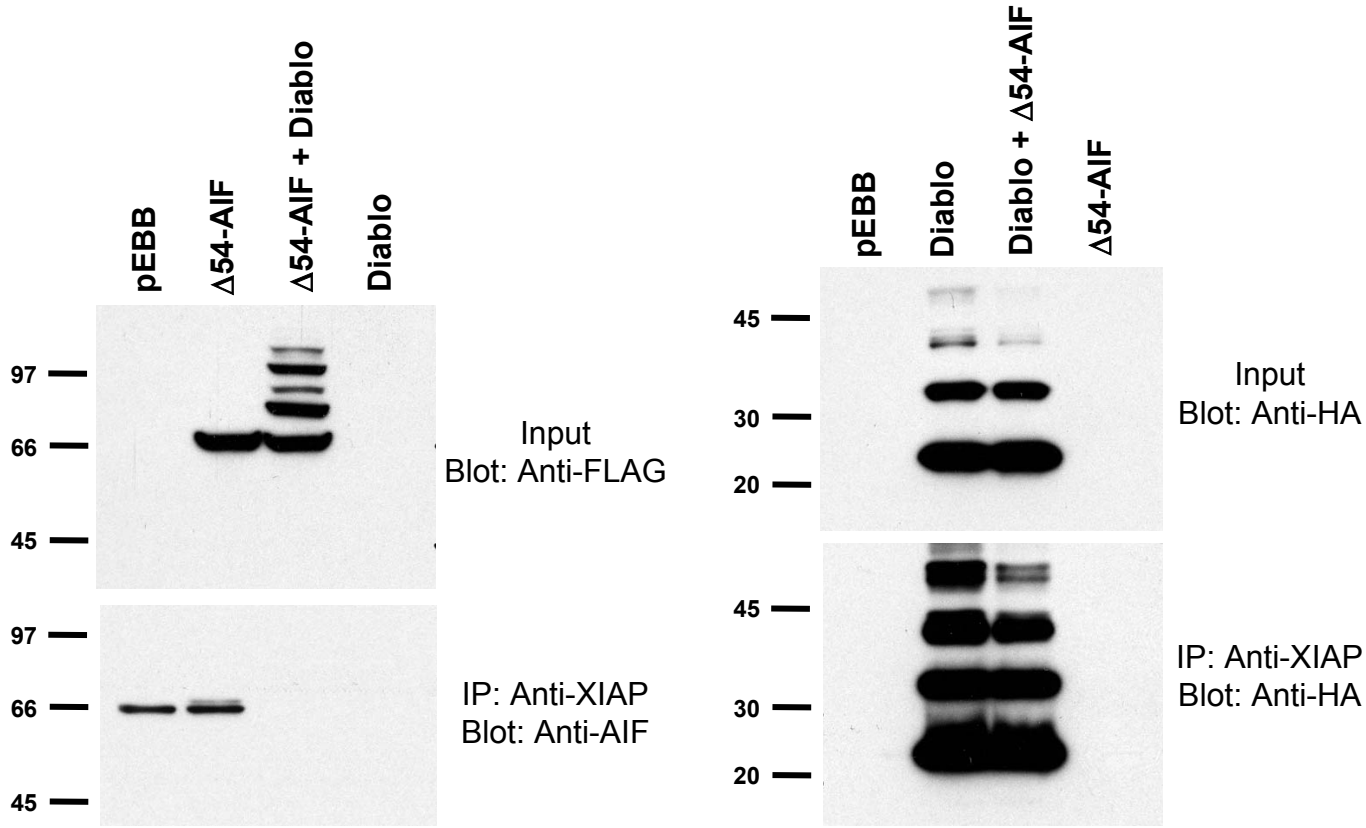

Figure 3: Smac/DIABLO disrupts XIAP-AIF association, increases AIF ubiquitination. HEK 293 cells were transiently transfected with a plasmid encoding XIAP in the absence and presence of Δ54-AIF-FLAG, Smac/DIABLO-HA, or both Δ54-AIF-FLAG and Smac/DIABLO-HA. Cell lysates were prepared and precipitated with an XIAP-specific antibody. Precipitated complexes were then immunoblotted for the presence of AIF (left panel) or Smac/DIABLO (right panel). Note the disruption of the association between XIAP and AIF as a result of Smac/DIABLO expression (left, lower panel), as well as the increase in AIF ubiquitination status following Smac/DIABLO co-expression (left, upper panel). The association between XIAP and Smac/DIABLO appears to be unaffected by the co-expression of AIF (right, upper panel).

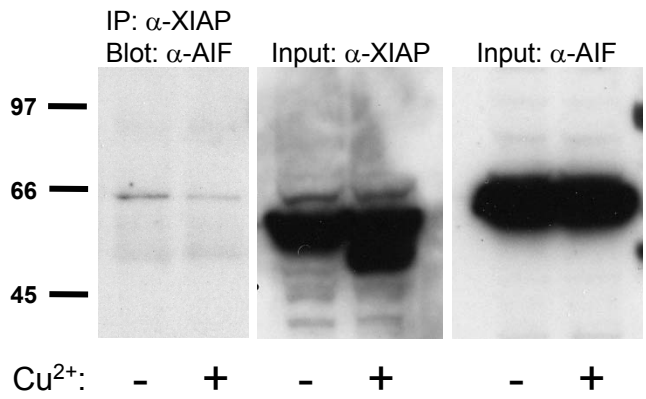

Figure 4: Copper shifted XIAP retains AIF binding capacity. HEK 293 were treated for 48 hours with copper sulfate in order to induce an electrophoretic mobility shift in XIAP (middle panel right lane), a phenomenon consistent with XIAP copper binding. Cells were then lysed, and XIAP was precipitated using an XIAP-specific antibody. The presence of AIF in precipitated material was then determined by immunoblotting. Note the precipitation of AIF (left panel) was unaffected by the copper-induced conformational change in XIAP. Also note that no change in AIF protein levels was observed following copper treatment (right panel).

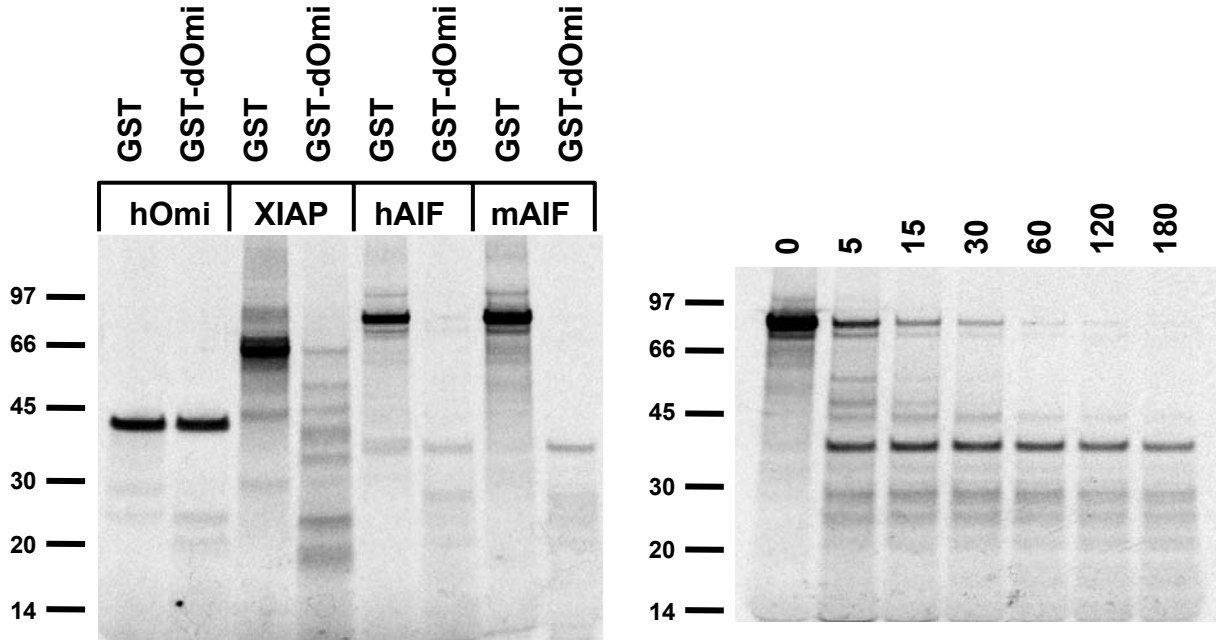

Figure 5: AIF is a substrate for Omi/Htra2 proteolysis. Left panel: ^{35}S-labeled, in vitro translated human Omi, XIAP, human AIF, and mouse AIF were incubated in the presence of recombinant glutathione-S-transferase (GST), or GST-tagged drosophila Omi for 4 h at 37 °C. The cleavage of substrate proteins was then determined following SDS-PAGE and autoradiography. Note that GST-Omi was able to induce cleavage in all substrates but Omi itself. Right panel: human AIF was incubated with GST-Omi at 37 °C for various amounts of time from 0 to 180 minutes. The cleavage of AIF was then determined by SDS-PAGE followed by autoradiography.

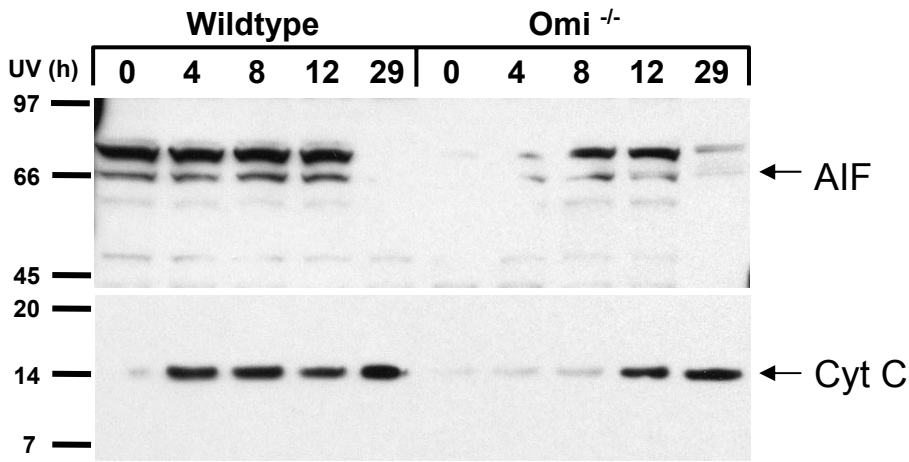

Figure 6: AIF release is unaffected, cytochrome *c* release is delayed in Omi knockout cells. Murine embryonic fibroblasts (MEFs) from wildtype or Omi knockout mice were treated with ultraviolet radiation. Cytoplasmic extracts were then prepared at various amounts of time following treatment, and the presence of both AIF (top panel) and cytochrome *c* (bottom panel) in the cytoplasm was determined by immunoblotting. Note that whereas the release of AIF appears unaffected (compare band intensity at 8 and 12 hours, ignoring transfer bubble present at 0 and 4 hours), the release of cytochrome *c* from Omi knockout cells is delayed when compared to wildtype controls.

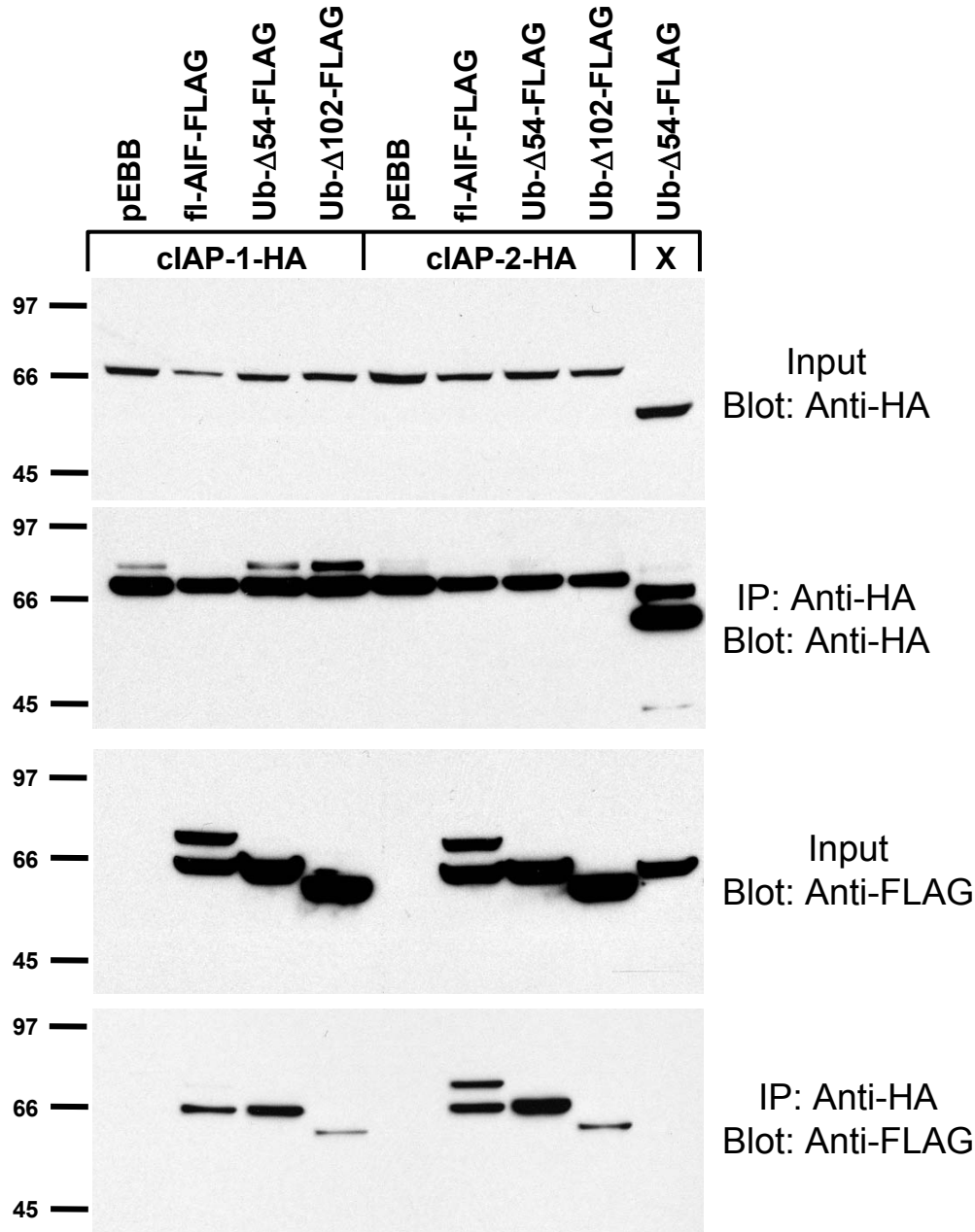

Figure 7: AIF binds the XIAP homologues cIAP-1 and cIAP-2. HEK 293 cells were transiently transfected with plasmids encoding HA-tagged cIAP-1 or cIAP-2 along with plasmids encoding full-length AIF-FLAG, Δ54-AIF-FLAG, or Δ102-AIF-FLAG. As a control, an additional sample was included in which cells were transfected with HA-tagged XIAP along with Δ54-AIF-FLAG. Cell lysates were prepared and IAP proteins were precipitated with anti-HA antibodies. The presence of AIF variants in immune complexes was determined by immunoblotting with a FLAG-specific antibody (bottom panel). Equivalent expression of all transfected proteins was determined by HA (top panel) and FLAG (lower middle panel) specific immunoblotting of input lysates, and precipitation of IAP proteins was confirmed by immunoblotting recovered material with an HA-specific antibody (upper middle panel). Note that all three AIF variants were efficiently precipitated by both cIAP-1 and cIAP-2, to an extent that was greater than that for XIAP.